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WO 01/20011 A1

(54) Title: DNA CONSTRUCT AND ITS USE

(57) Abstract: A DNA construct comprising in the 5' to 3' direction of transcription operably linked a promoter region directing transcription to the seed of an oilseed plant, a nucleotide sequence coding for at least one peptide with enzyme activity necessary for keto group containing xanthophyll production and esterification in an oilseed plant and a transcriptional termination region is disclosed. The DNA construct may additionally comprise a nucleotide sequence coding for a transit peptide directing the translated fusion polypeptide to the chloroplast of the oilseed plant. The peptide with enzyme activity is preferably a peptide with β -carotene C-4-oxygenase activity, e.g. from the alga *Chlamydomonas reinhardtii*. Comprised by the invention are also a transgenic oilseed plant cell, e.g. of rape, sunflower, soybean or mustard origin, and a transgenic oilseed plant-produced xanthophyll, such as canthaxanthin or astaxanthin, and also astaxanthin esters.

DNA construct and its use.

The present invention relates to a new DNA construct for transformation into oilseed plants. The DNA construct comprises nucleotide sequences encoding peptides with enzyme activities necessary for the high-level production and esterification of keto group-containing xanthophylls in oilseed plants.

Background of the invention

Carotenoids are produced *de novo* by plants, fungi, algae and some bacteria. A number of biosynthetic steps are needed for the biological production of the carotenoids. There are two chemically different groups of carotenoids, namely carotenes containing only carbon and hydrogen molecules and xanthophylls containing oxygen in the molecule in addition to carbon and hydrogen.

The xanthophylls, and particularly astaxanthin (3,3'-dihydroxy- β - β -carotene-4,4'-dione), are often colored pigments and are used as such or as anti-oxidants.

Carotenes are biological precursors for the production of the oxygen-containing xanthophylls. There are two types of enzymes responsible for the introduction of hydroxy groups and keto groups into the carotenes, namely hydroxylases and ketolases, respectively.

The keto group-containing xanthophyll astaxanthin, which has keto and hydroxy groups, is biosynthetically produced from beta-carotene.

Large-scale production of xanthophylls from natural sources is at present performed by AstaCarotene AB, Gustavsberg, Sweden, by cultivation of the alga *Haematococcus pluvialis* for the production of astaxanthin in esterified form.

It would be desirable to be able to produce keto group-containing xanthophylls particularly astaxanthin, in oilseed plants. Oilseed plants have naturally β -carotene hydroxylases but lack β -carotene C-4-oxygenase enzymes or ketolases.

Description of the invention

The present invention provides DNA constructs enabling and promoting production of keto group containing xanthophylls, especially astaxanthin, in oilseed plants, such as rape, sunflower, soybean and mustard. The DNA construct is transformed into the oilseed plant cell for expression of a protein or fused protein which has an enzyme activity enabling keto group insertion into a carotene or hydroxy carotene for the biosynthetic production of a keto group containing xanthophyll, such as cantaxanthin (β , β -carotene-4,4'-dione) and/or astaxanthin. Use is thus made of the biosynthetic pathway of the oilseed plant to

produce carotenoids. The naturally occurring synthesis of carotenoids involves a number of enzymes, namely 1-D-deoxyxylulose 5-phosphate synthase, isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase, geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, zeta-carotene desaturase, lycopene beta-cyclase, β -carotene hydroxylase, and β -carotene C-4-oxygenase. Genes coding for peptides having these enzymatic activities may be inserted into the DNA construct of the invention, one or several per construct, to promote high-level production in the transgenic oilseed plant. In case only one enzyme coding gene is inserted per plant, two or more plants may be sexually interbred to produce plants containing all the desired enzyme activities.

Thus, the present invention is directed to a DNA construct comprising in the 5' to 3' direction of transcription operably linked a promoter region directing transcription to the seed of an oilseed plant, a nucleotide sequence coding for at least one peptide with enzyme activity necessary for keto group containing xanthophyll production and esterification in an oilseed plant and a transcriptional termination region.

In a preferred embodiment of the invention the DNA construct additionally comprises between the promoter region and the nucleotide sequence coding for at least one peptide with enzyme activity a nucleotide sequence coding for a transit peptide directing the translated fusion polypeptide to the chloroplast of the oilseed plant.

The DNA construct is preferably such that the promoter is a napin promoter, the peptide with enzyme activity necessary for keto group containing xanthophyll production is selected from the group consisting of peptides with 1-D-deoxyxylulose 5-phosphate synthase, isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase, geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, zeta-carotene desaturase, lycopene beta-cyclase, β -carotene hydroxylase, and β -carotene C-4-oxygenase activity. To promote esterification of astaxanthin a nucleotide sequence coding for a peptide with acyl transferase activity may be included in the group.

In a preferred embodiment of the DNA construct according to the invention the nucleotide sequence coding for a peptide with enzyme activity is a nucleotide sequence coding for a N-terminally truncated β -carotene C-4-oxygenase gene from the alga *Haematococcus pluvialis*.

An example of the DNA construct of the invention is presented in the sequence listing as SEQ ID NO:1 and in Fig.1.

The present invention is also directed to a transgenic oilseed plant cell comprising the DNA construct of the invention, and preferably the oilseed plant is selected from the group consisting of rape, sunflower, soybean and mustard.

The invention is additionally directed to transgenic oilseed plant-produced xanthophyll, e.g. canthaxanthin and astaxanthin.

A preferred aspect of the invention is directed to transgenic oilseed plant-produced astaxanthin esters.

The present invention will now be illustrated with reference to the DNA construct disclosed in the sequence listing and in Fig.1, and the following description of embodiments. However, the invention is not limited to these exemplifications.

Short description of the drawings

Fig.1 illustrates the nucleotide sequence of the DNA construct comprising the napin promoter, the chloroplast localization signal, the N-terminally truncated β -carotene C-4-oxygenase gene and the termination sequence, and the deduced amino acid sequences of the transit peptide and the β -carotene C-4-oxygenase.

Description of embodiments

The invention is illustrated by production of astaxanthin in the seed of oilseed rape. The astaxanthin produced in the seed of the transgenic plant is extracted as part of the extracted oil. By use of conventionally used protocols for *Agrobacterium tumefaciens* mediated transformation such as described by (Hoekema et al.1983, An et al. 1986, Fry et al. 1987, DeBlock et al. 1988, Radke et al.1988, or Moloney et al. 1989) transgenic plants are produced having a chimeric DNA construct that is genetically inherited and is able to produce astaxanthin. The nucleotide sequence of the chimeric DNA construct consist of four parts of different genetic origin namely: (1) a promoter, (2) a localization signal, (3) a β -carotene C-4-oxygenase coding region and (4) a termination sequence.

The napin promoter directs transcription to the seed of oilseed rape (Stålberg et al 1996). This promoter was coupled to a localization signal similar but not identical to a transit peptide (TP) of Rbcs1a (Krebbers, 1988) that directs the translated product of a fused gene to the chloroplast. The promoter and the TP sequence were ligated to a part of the coding sequence of a ketolase gene BCK (Kajiwara et al. 1995). This enzyme oxygenates β -carotene to canthaxanthin, (Fraser et al. 1997). The chimeric DNA construct was then coupled to a suitable termination sequence, e.g. that of the *Agrobacterium tumefaciens* nopaline synthase gene (the nos 3' end)(Bevan et al. 1983), as illustrated in Fig.1.

Cellular storage of Astaxanthin

The storage of large amounts of free astaxanthin in plants will be difficult due to toxic effects of the molecule as it intercalates in the plant membranes. An effective esterification of astaxanthin to fatty acids enables storage of the esterified molecules in triacylglycerol containing oleosomes. Thus, an acyl transferase can be claimed to be of fundamental importance for the process, as is proteins that can mediate transport of different forms of astaxanthin from the chloroplast to the vesicles.

Sequences and oligonucleotides used in the construction of the DNA construct*1. Napin promoter (GeneBank ACCESSION No. J02798)*

This promoter sequence, a 1145 base pair fragment including the 5' leader sequence has a unique HindIII site at the 5' end. The 3' end was synthesized with an additionally 6 nucleotide BamHI site.

2. Transit peptide similar to RBCS1a (GeneBank ACCESSION No. X13611, X14565)

The transit peptide (TP) was amplified by PCR from -28 to the end of the transit cleavage aa=54/55 site of the Rbcs1a gene. The 5' end was synthesized with a BamHI site and similarly the 3' sequence was synthesized with a XbaI site. The two following oligonucleotides were used for the PCR amplification.

BamHI

5' primer: TP1 5'AGAC GGATCC TCAGTCACACAAAGAGTA 3'

SacI XbaI

3' primer: TP2 5'GTTC GAGCTC TCTAGA CATGCAGTTAACGC 3'

3. BCK (β -carotene C-4 oxygenase) (Genebank ACCESSION No. D45881)

The BCK fragment was amplified by PCR including a 5' XbaI site and was ligated to the TP already described. The 5' primer (BCK1) used for PCR, is homologous to the BCK sequence from nucleotide 264 and the 3' oligonucleotide (Ax40) ends with a stop codon and was synthesized with a SacI restriction site for cloning. The synthesized fragment was fused to the TP as shown in Fig 1.

Oligonucleotides used for PCR:

XbaI

5' primer: BCK1 5'ACAG TCTAGA ATGCCATCCGAGTCGTCA 3'

SacI

3'primer: AX40 5'CACCGAGCTCCATGACACTCTTGTGCAGA 3'

Description of SEQ ID NO:1 and SEQ ID NO:2

The sequences shown i Fig.1 are the same as the two sequences which are shown in the sequence listing.

The SEQ ID NO:1 is a nucleotide sequence composed of the following features:

	Nucleotide No.
5	Cloning site HindIII 1-6
	Napin Promoter 1-1145
	Cloning site BamHI 1146-1151
	Transit peptide leader 1152-1178
10	Transit peptide coding 1179-1347
	Cloning site XbaI 1348-1353
	β -carotene C-4-oxygenase 1354-2217
	β -carotene C-4-oxygenase 3' untranslated 2218-2266
	Cloning site SacI 2267-2272
15	Nopaline synthetase termination 2273-2536
	Cloning site EcoRI 2538-2543

The SEQ ID NO: 2 is a deduced amino acid sequence of the fusion protein of the transit peptide and the peptide with β -carotene C-4-oxygenase activity.

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5 Arabidopsis-thaliana using a binary vector system. Plant Physiology 81 (1) 301-305.
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Claims

1. A DNA construct comprising in the 5' to 3' direction of transcription operably linked a promoter region directing transcription to the seed of an oilseed plant, a nucleotide sequence coding for at least one peptide with enzyme activity necessary for keto group
5 containing xanthophyll production and esterification in an oilseed plant and a transcriptional termination region.
2. The DNA construct according to claim 1, which between the promoter region and the nucleotide sequence coding for at least one peptide with enzyme activity additionally comprises a nucleotide sequence coding for a transit peptide directing the translated fusion
10 polypeptide to the chloroplast of the oilseed plant.
3. The DNA construct according to claim 1 or 2, wherein the promoter is a napin promoter, the peptide with enzyme activity necessary for keto group containing xanthophyll production and esterification is selected from the group consisting of peptides with, 1-D-deoxyxylulose 5-phosphate synthase, isopentenyl pyrophosphate:dimethylallyl pyrophosphate
15 isomerase, geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, zeta-carotene desaturase, lycopene beta-cyclase, β -carotene hydroxylase, β -carotene C-4-oxygenase, and acyl transferase activity.
4. The DNA construct according to any one of claims 1 - 3, wherein the nucleotide sequence coding for a peptide with enzyme activity is a nucleotide sequence
20 coding for a N-terminally truncated β -carotene C-4-oxygenase gene from the alga *Haematococcus pluvialis*.
5. The DNA construct according to claim 4, wherein the nucleotide sequence is SEQ ID NO:1.
6. Transgenic oilseed plant cell comprising the DNA construct of any one of
25 claims 1-5 .
7. Transgenic oilseed plant cell according to claim 6, wherein the oilseed plant is selected from the group consisting of rape, sunflower, soybean and mustard.
8. Transgenic oilseed plant-produced xanthophyll.
9. Transgenic oilseed plant-produced xanthophyll according to claim 8, wherein
30 the xanthophyll is canthaxanthin
10. Transgenic oilseed plant-produced xanthophyll according to claim 8, wherein the xanthophyll is astaxanthin.
11. Transgenic oilseed plant-produced xanthophyll according to claim 8, wherein the xanthophyll is astaxanthin esters.

1/3

Napin promoter

napin promoter
AAGCTTTCTTCATCGGTGATTGATTCCTTTAAAGACTTATGTTTCTTATCTTGCTTCTGA
GGCAAGTATTCAGTTACCAGTTACCACTTATATTCTGGACTTTCTGACTGCATCCTCATT
TTTCCAACATTTTAAATTTCACTATTGGCTGAATGCTTCTTCTTTGAGGAAGAAACAATT
CAGATGGCAGAAATGTATCAACCAATGCATATATACAAATGTACCTCTTGTTCTCAAAC
ATCTATCGGATGGTTCATTGCTTTGTTCATCCAATTAGTGACTACTTTATATTATTCAC
TCCTCTTTATACTATTTTCATGCGAGGTTGCCATGTACATTATATTTGTAAGGATTGAC
GCTATTGAGCGTTTTTCTTCAATTTTCTTTATTTTAGACATGGGTATGAAATGTGTGTTA
GAGTTGGGTGAATGAGATATACGTTCAAGTGAAGTGGCATAACCGTTCTCGAGTAAGGAT
GACCTACCCATTCTTGAGACAAATGTTACATTTTAGTATCAGAGTAAAATGTGTACCTAT
AACTCAAATTCGATTGACATGTATCCATTCAACATAAAATTAAACCAGCCTGCACCTGCA
TCCACATTTCAAGTATTTTCAAACCGTTCGGCTCCTATCCACCGGGTGTAACAAGACGGA
TTCCGAATTTGGAAGATTTTGACTCAAATCCCAATTTATATTGACCGTGACTAAATCAA
CTTTAACTTCTATAATTCTGATTAAGCTCCCAATTTATATTCCCAACGGCACTACCTCCA
AAATTTATAGACTCTCATCCCCTTTTAAACCAACTTAGTAAACGTTTTTTTTTTTAAATTT
TATGAAGTTAAGTTTTTACCTTGTTTTTAAAAAGAATCGTTCATAAGATGCCATGCCAGA
ACATTAGCTACACGTTACACATAGCATGCAGCCGCGGAGAATTGTTTTTCTTCGCCACTT
GTCACTCCCTTCAAACACCTAAGAGCTTCTCTCTCACAGCACACACATACAATCACATGC
GTGCATGCATTATTACACGTGATCGCCATGCAAATCTCCTTTATAGCCTATAAATTAAC
TATCCGCTTCACTCTTTACTCAAACCAAACTCATCAATACAAACAAGATTAAAAACATA

End -28 untranslated leader TP start

CACGAGGATCCTCAGTCACACAAAGAGTAAAGAAGAACAATGGCTTCCTCTATGCTCTCT
M A S S M L S

TCCGCTACTATGGTTGCCTCTCCGGCTCAGGCCACTATGGTCGCTCCTTTCAACGGACTT
S A T M V A S P A Q A T M V A P F N G L

AAGTCCTCCGCTGCCTTCCCAGCCACCCGCAAGGCTAACCAACGACATTACTTCCATCACA
K S S A A F P A T R K A N N D I T S I T

FIG. 1

2/3

TP End C-4-Oxygenase
AGCAACGGCGGACGCGTTAACTGCATGTCTAGAATGCCATCCGAGTCGTCAGACGCAGCT
S N G G R V N C M S R M P S E S S D A A
CGTCCTGCGCTAAAGCACGCCTACAAACCTCCAGCATCTGACGCCAAGGGCATCACGATG
R P A L K H A Y K P P A S D A K G I T M
GCGCTGACCATCATTGGCACCTGGACCGCAGTGTTTTTACACGCAATATTTCAAATCAGG
A L T I I G T W T A V F L H A I F Q I R
CTACCGACATCCATGGACCAGCTTCACTGGTTGCCTGTGTCCGAAGCCACAGCCCAGCTT
L P T S M D Q L H W L P V S E A T A Q L
TTGGGCGGAAGCAGCAGCCTACTGCACATCGCTGCAGTCTTCATTGTACTTGAGTTCTCTG
L G G S S S L L H I A A V F I V L E F L
TACACTGGTCTATTCATCACCACACATGACGCAATGCATGGCACCATAGCTTTGAGGCAC
Y T G L F I T T H D A M H G T I A L R H
AGGCAGCTCAATGATCTCCTTGGCAACATCTGCATATCACTGTACGCCTGGTTTGACTAC
R Q L N D L L G N I C I S L Y A W F D Y
AGCATGCTGCATCGCAAGCACTGGGAGCACCACAACCATACTGGCGAAGTGGGGAAAGAC
S M L H R K H W E H H N H T G E V G K D
CCTGACTTCCACAAGGGAAATCCCGGCCTTGTCCCCTGGTTCGCCAGCTTCATGTCCAGC
P D F H K G N P G L V P W F A S F M S S
TACATGTCCCTGTGGCAGTTTGCCCGGCTGGCATGGTGGGCAGTGGTGATGCAAATGCTG
Y M S L W Q F A R L A W W A V V M Q M L
GGGGCGCCCATGGCAAATCTCCTAGTCTTCATGGCTGCAGCCCCAATCTTGTGAGCATTC
G A P M A N L L V F M A A A P I L S A F
CGCCTCTTCTACTTCGGCACTTACCTGCCACACAAGCCTGAGCCAGGCCCTGCAGCAGGC
R L F Y F G T Y L P H K P E P G P A A G
TCTCAGGTGATGGCCTGGTTCAGGGCCAAGACAAGTGAGGCATCTGATGTGATGAGTTTC
S Q V M A W F R A K T S E A S D V M S F
CTGACATGCTACCACTTTGACCTGCACTGGGAGCACCACAGATGGCCCTTTGCCCCCTGG
L T C Y H F D L H W E H H R W P F A P W
C-4 oxygenase Stop
TGGCAGCTGCCCCACTGCCGCCGCTGTCCGGGCGTGGCCTGGTGCCTGCCTTGGCATGA
W Q L P H C R R L S G R G L V P A L A *

FIG.1 (cont.)

3/3

C-4 oxygenase untranslated region Nos term
CCTGGTCCCTCCGCTGGTGACCCAGCGTCTGCACAAGAGTGTTCATGGAGCTCGAATTTCC

CCGATCGTTCAAACATTTGGCAATAAAGTTTTCTTAAGATTGAATCCTGTTGCCGGTCTTG

CGATGATTATCATATAATTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAAT

GCATGACGTTATTTATGAGATGGGTTTTTATGATTAGAGTCCC GCAATTATACATTTAAT

ACGCGATAGAAAACAAATATAGCGCGCAA A CTAGGATAAATTATCGCGCGCGGTGTCAT
 end
CTATGTTACTAGATCGGGAATTC

Fig.1 (cont.)

SEQUENCE LISTING

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<120> DNA construct and its use

<130> 29295-AstaCarotene

<140>

<141>

<160> 2

<170> PatentIn Ver. 2.1

<210> 1

<211> 2543

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: napin promoter
+ chloroplast localization signal + beta-carotene C-4 oxygenase
coding sequence + termination sequence

<220>

<221> promoter

<222> (1) .. (1145)

<220>

<221> transit_peptide

<222> (1179) .. (1347)

<220>

<221> CDS

<222> (1179) .. (2217)

<220>

<221> terminator

<222> (2273) .. (2536)

<400> 1

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ggcaagtatt cagttaccag ttaccactta tattctggac tttctgactg catcctcatt 120
tttccaacat tttaaatttc actattggct gaatgcttct tctttgagga agaaacaatt 180
cagatggcag aaatgtatca accaatgcat atatacaaat gtacctcttg ttctcaaaac 240
atctatcgga tgggtccatt tgctttgtca tccaattagt gactacttta tattattcac 300
tcctctttat tactattttc atgcgagggt gccatgtaca ttatatttgt aaggattgac 360
gctattgagc gtttttcttc aattttcttt atttttagaca tgggtatgaa atgtgtgtta 420
gagttgggtt gaatgagata tacgttcaag tgaagtggca taccgttctc gagtaaggat 480
gacctaccca ttcttgagac aaatgttaca ttttagtatc agagtaaaat gtgtacctat 540

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gtgcatgcat	tattacacgt	gatcgccatg	caaatctcct	ttatagccta	taaattaact	1080
catccgcttc	actcttttact	caaaccaaaa	ctcatcaata	caaacaagat	taaaaaacata	1140
cacgaggatc	ctcagtcaca	caaagagtaa	agaagaaca	atg gct tcc tct atg		1194
				Met Ala Ser Ser Met		
				1 5		
ctc tct tcc gct act atg gtt gcc tct ccg gct cag gcc act atg gtc						1242
Leu Ser Ser Ala Thr Met Val Ala Ser Pro Ala Gln Ala Thr Met Val						
		10		15		20
gct cct ttc aac gga ctt aag tcc tcc gct gcc ttc cca gcc acc cgc						1290
Ala Pro Phe Asn Gly Leu Lys Ser Ser Ala Ala Phe Pro Ala Thr Arg						
		25		30		35
aag gct aac aac gac att act tcc atc aca agc aac ggc gga cgc gtt						1338
Lys Ala Asn Asn Asp Ile Thr Ser Ile Thr Ser Asn Gly Gly Arg Val						
		40		45		50
aac tgc atg tct aga atg cca tcc gag tcg tca gac gca gct cgt cct						1386
Asn Cys Met Ser Arg Met Pro Ser Glu Ser Ser Asp Ala Ala Arg Pro						
		55		60		65
gcg cta aag cac gcc tac aaa cct cca gca tct gac gcc aag ggc atc						1434
Ala Leu Lys His Ala Tyr Lys Pro Pro Ala Ser Asp Ala Lys Gly Ile						
		70		75		80
acg atg gcg ctg acc atc att ggc acc tgg acc gca gtg ttt tta cac						1482
Thr Met Ala Leu Thr Ile Ile Gly Thr Trp Thr Ala Val Phe Leu His						
		90		95		100
gca ata ttt caa atc agg cta ccg aca tcc atg gac cag ctt cac tgg						1530
Ala Ile Phe Gln Ile Arg Leu Pro Thr Ser Met Asp Gln Leu His Trp						
		105		110		115
ttg cct gtg tcc gaa gcc aca gcc cag ctt ttg ggc gga agc agc agc						1578
Leu Pro Val Ser Glu Ala Thr Ala Gln Leu Leu Gly Gly Ser Ser Ser						
		120		125		130
cta ctg cac atc gct gca gtc ttc att gta ctt gag ttc ctg tac act						1626
Leu Leu His Ile Ala Ala Val Phe Ile Val Leu Leu Glu Phe Leu Tyr Thr						
		135		140		145

ggt cta ttc atc acc aca cat gac gca atg cat ggc acc ata gct ttg 1674
 Gly Leu Phe Ile Thr Thr His Asp Ala Met His Gly Thr Ile Ala Leu
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 agg cac agg cag ctc aat gat ctc ctt ggc aac atc tgc ata tca ctg 1722
 Arg His Arg Gln Leu Asn Asp Leu Leu Gly Asn Ile Cys Ile Ser Leu
 170 175 180
 tac gcc tgg ttt gac tac agc atg ctg cat cgc aag cac tgg gag cac 1770
 Tyr Ala Trp Phe Asp Tyr Ser Met Leu His Arg Lys His Trp Glu His
 185 190 195
 cac aac cat act ggc gaa gtg ggg aaa gac cct gac ttc cac aag gga 1818
 His Asn His Thr Gly Glu Val Gly Lys Asp Pro Asp Phe His Lys Gly
 200 205 210
 aat ccc ggc ctt gtc ccc tgg ttc gcc agc ttc atg tcc agc tac atg 1866
 Asn Pro Gly Leu Val Pro Trp Phe Ala Ser Phe Met Ser Ser Tyr Met
 215 220 225
 tcc ctg tgg cag ttt gcc cgg ctg gca tgg tgg gca gtg gtg atg caa 1914
 Ser Leu Trp Gln Phe Ala Arg Leu Ala Trp Trp Ala Val Val Met Gln
 230 235 240 245
 atg ctg ggg gcg ccc atg gca aat ctc cta gtc ttc atg gct gca gcc 1962
 Met Leu Gly Ala Pro Met Ala Asn Leu Leu Val Phe Met Ala Ala Ala
 250 255 260
 cca atc ttg tca gca ttc cgc ctc ttc tac ttc ggc act tac ctg cca 2010
 Pro Ile Leu Ser Ala Phe Arg Leu Phe Tyr Phe Gly Thr Tyr Leu Pro
 265 270 275
 cac aag cct gag cca ggc cct gca gca ggc tct cag gtg atg gcc tgg 2058
 His Lys Pro Glu Pro Gly Pro Ala Ala Gly Ser Gln Val Met Ala Trp
 280 285 290
 ttc agg gcc aag aca agt gag gca tct gat gtg atg agt ttc ctg aca 2106
 Phe Arg Ala Lys Thr Ser Glu Ala Ser Asp Val Met Ser Phe Leu Thr
 295 300 305
 tgc tac cac ttt gac ctg cac tgg gag cac cac aga tgg ccc ttt gcc 2154
 Cys Tyr His Phe Asp Leu His Trp Glu His His Arg Trp Pro Phe Ala
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 Pro Trp Trp Gln Leu Pro His Cys Arg Arg Leu Ser Gly Arg Gly Leu
 330 335 340
 gtg cct gcc ttg gca tgacctgggc cctccgctgg tgaccagcg tctgcacaag 2257
 Val Pro Ala Leu Ala
 345
 agtgtcatgg agctcgaatt tccccgatcg ttcaaacatt tggcaataaa gtttcttaag 2317
 attgaatcct gttgccggtc ttgcgatgat tatcatataa tttctgttga attacgttaa 2377
 gcatgtaata attaacaatgt aatgcatgac gttatattatg agatggggttt ttatgattag 2437
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<210> 2
 <211> 346
 <212> PRT
 <213> Artificial Sequence
 <223> Description of Artificial Sequence: deduced fusion protein of
 transit peptide + peptide with beta-carotene C-4 oxygenase activity

<400> 2

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Met Ala Ser Ser Met Leu Ser Ser Ala Thr Met Val Ala Ser Pro Ala
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      20             25             30
Phe Pro Ala Thr Arg Lys Ala Asn Asn Asp Ile Thr Ser Ile Thr Ser
      35             40             45
Asn Gly Gly Arg Val Asn Cys Met Ser Arg Met Pro Ser Glu Ser Ser
      50             55             60
Asp Ala Ala Arg Pro Ala Leu Lys His Ala Tyr Lys Pro Pro Ala Ser
      65             70             75             80
Asp Ala Lys Gly Ile Thr Met Ala Leu Thr Ile Ile Gly Thr Trp Thr
      85             90             95
Ala Val Phe Leu His Ala Ile Phe Gln Ile Arg Leu Pro Thr Ser Met
      100            105            110
Asp Gln Leu His Trp Leu Pro Val Ser Glu Ala Thr Ala Gln Leu Leu
      115            120            125
Gly Gly Ser Ser Ser Leu Leu His Ile Ala Ala Val Phe Ile Val Leu
      130            135            140
Glu Phe Leu Tyr Thr Gly Leu Phe Ile Thr Thr His Asp Ala Met His
      145            150            155            160
Gly Thr Ile Ala Leu Arg His Arg Gln Leu Asn Asp Leu Leu Gly Asn
      165            170            175
Ile Cys Ile Ser Leu Tyr Ala Trp Phe Asp Tyr Ser Met Leu His Arg
      180            185            190
Lys His Trp Glu His His Asn His Thr Gly Glu Val Gly Lys Asp Pro
      195            200            205
Asp Phe His Lys Gly Asn Pro Gly Leu Val Pro Trp Phe Ala Ser Phe
      210            215            220
Met Ser Ser Tyr Met Ser Leu Trp Gln Phe Ala Arg Leu Ala Trp Trp
      225            230            235            240
Ala Val Val Met Gln Met Leu Gly Ala Pro Met Ala Asn Leu Leu Val
      245            250            255

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Phe Met Ala Ala Ala Pro Ile Leu Ser Ala Phe Arg Leu Phe Tyr Phe
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Gly Thr Tyr Leu Pro His Lys Pro Glu Pro Gly Pro Ala Ala Gly Ser
275 280 285

Gln Val Met Ala Trp Phe Arg Ala Lys Thr Ser Glu Ala Ser Asp Val
290 295 300

Met Ser Phe Leu Thr Cys Tyr His Phe Asp Leu His Trp Glu His His
305 310 315 320

Arg Trp Pro Phe Ala Pro Trp Trp Gln Leu Pro His Cys Arg Arg Leu
325 330 335

Ser Gly Arg Gly Leu Val Pro Ala Leu Ala
340 345

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 00/01767

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12N 15/82, C12N 9/02, C12N 9/10, A01H 5/00, C12P 23/00 // (C12N 9/02, C12R 1:89)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12N, C12P, A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,OK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9907867 A1 (CALGENE LLC), 18 February 1999 (18.02.99), see abstract, page 13, lines 15-23, claims --	1-11
X	WO 9806862 A1 (CALGENE, INC.), 19 February 1998 (19.02.98), see page 8. line 9 - page 12, line 15; page 13, line 22 - page 15, line 5 --	1-11
X	Susan Budavari et al "THE MERCK INDEX", twelfth edition", 1996, MERCK & CO., INC. NJ, see entries 890, "Astaxanthin"; 1798, "Canthaxanthin"; 10197, "Xanthophyll". --	8-10

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

12 December 2000

Date of mailing of the international search report

- 20 -12- 2000

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 00/01767

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9818910 A1 (YISSUM RESEARCH AND DEVELOPMENT COMPANY OF THE HEBREW UNIVERSITY OF JERUSALEM), 7 May 1998 (07.05.98), see abstract, page 28, line 24 - page 29, line 4	1-4
A	--	5-11
A	WO 9613149 A1 (AMOCO CORPORATION), 9 May 1996 (09.05.96)	1-11
A	--	
A	EMBL/GenBank/DDBJ databases, accession no. X86782, 1997-11-30, Harker M. et al: "H.pluvialis mRNA for beta-carotene C-4 oxygenase"	4,5
A	--	
A	EMBL/GenBank/DDBJ databases, accession no. D45881, 1995-12-29, Kajiwarea S.: "Haematococcus pluvialis mRNA for bet-carotene ketolase, complete cds"	3
A	--	
A	EMBL/GenBank/DDBJ databases, accession no. X86783, 1998-06-02, Harker M. et al: "H.pluvialis mRNA for phyteone desaturase"	3
A	--	
A	EMBL/GenBank/DDBJ databases, accession no. AF082325, Sun Z. et al: "Haematococcus pluvialis isopenetyl pyrophosphate:dimethylallyl pyrophosphate isomerase (ipiHp1) mRNA, complete cd, 1998-08-18	3
X	--	
X	EMBL/GenBank/DDBJ databases, accession no. AF082326, 1998-08-18, Sun Z. et al: "Haematococcus pluvialis isopenetyl pyrophosphate:dimethylallyl pyrophosphate isomerase (ipiHp2) mRNA, complete cds"	3
	--	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 00/01767

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EMBL/GenBank/DDBJ databases, accession no. AF162276, 1999-09-10, Linden H.: "Haematococcus pluvialis carotenoid hydroxylase mRNA, partial cds" --	3
A	WO 9930701 A1 (ASTACAROTENE), 24 June 1999 (24.06.99), see abstract and claims --	11
A	WO 9837874 A1 (ASTACAROTENE AB), 3 Sept 1998 (03.09.98), see abstract and claims --	11
A	JOURNAL OF PHOTOCHEMISTRY AND PHOTOBIOLOGY B, Volume 30, 1995, BISWAL, B et al, "Carotenoid catabolism during leaf senescence and its control by light" page 3 - page 13 -- -----	11

INTERNATIONAL SEARCH REPORT

International application No.
SE00/01767

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see extra sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
SE00/01767

According to Article 34 (3) (a-c) and Rule 13.2, an international application shall relate to one invention only or to a group of inventions linked by one or more of the same or corresponding "special technical features", i.e. features that define a contribution which each of the inventions makes over the prior art. The present application relates to five such groups of inventions, namely:

1. A DNA construct encoding an enzyme in the carotenoid biosynthetic pathway and cells expressing the enzyme, according to claims 1-7.
2. Transgenic oilseed plant-produced xanthophyll, according to claim 8.
3. Transgenic oilseed plant-produced canthaxanthin, according to claim 9.
4. Transgenic oilseed plant-produced astaxanthin, according to claim 10.
5. Transgenic oilseed plant-produced astaxanthin esters, according to claim 11.

The feature common to all inventions is the transgenic production of carotenoids in oilseed plants. However, this feature is already known through WO-A1-9806862. The production of different carotenoids, and DNA constructs facilitating the production, is thus not linked by a special technical feature as required by Rule 13.2. As the additional effort of searching inventions 2-5 does not justify an additional search fee, all inventions have been searched.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/SE 00/01767

Patent document cited in search report			Publication date	Patent family member(s)		Publication date
WO	9907867	A1	18/02/99	AU	8900298 A	01/03/99
				EP	1002117 A	24/05/00
WO	9806862	A1	19/02/98	AU	4058497 A	06/03/98
				BR	9713462 A	28/03/00
				CN	1227609 A	01/09/99
				EP	0925366 A	30/06/99
WO	9818910	A1	07/05/98	AU	4743697 A	22/05/98
				NO	991996 A	22/06/99
				US	5916791 A	29/06/99
				US	5965795 A	12/10/99
				CN	1247565 A	15/03/00
				EP	0951534 A	27/10/99
				PL	332965 A	25/10/99
WO	9613149	A1	09/05/96	AU	697358 B	01/10/98
				AU	3970195 A	23/05/96
				CA	2203815 A	09/05/96
				CN	1172416 A	04/02/98
				EP	0792352 A	03/09/97
				JP	10509309 T	14/09/98
				NO	971945 A	27/06/97
				NZ	296012 A	28/05/99
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				US	5618988 A	08/04/97
WO	9930701	A1	24/06/99	AU	1897299 A	05/07/99
				EP	1049460 A	08/11/00
				NO	20003042 A	14/06/00
				SE	511237 C	30/08/99
				SE	9704693 A	17/06/99
WO	9837874	A1	03/09/98	AU	719090 B	04/05/00
				AU	2796797 A	19/11/97
				AU	6295198 A	18/09/98
				CN	1248912 T	29/03/00
				EP	0898823 A	03/03/99
				EP	0981338 A	01/03/00
				NO	994109 A	27/10/99
				PL	335370 A	25/04/00
				SE	9700708 A	28/08/98

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